Antibody targeted delivery of prodrug converting enzymes using protein nanoparticle platform for HER2-positive breast cancer therapy

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Sophia Guldberg

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Approved by:
Dr. M.G. Finn, *Advisor*
School of Chemistry and Biochemistry
Georgia Institute of Technology

x._____

Dr. Loren Williams
School of Chemistry and Biochemistry
Georgia Institute of Technology

x._____

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Abstract

Approximately 1 in 8 women in the United States will be diagnosed with breast cancer.[1] Among these women, 25-30% will have breast cancer where the *HER2* gene is overexpressed and human epidermal growth factor receptor 2 (HER2) is overexpressed, which increases the aggressiveness of the cancer.[2] The aggressiveness of HER2-positive breast cancer results in decreased long-term survival. For this reason, new HER-2 targeted therapies need to be developed to increase remission and survival of HER2-postive breast cancer patients. A notable success in this field has been the use of Genentech's anti-HER2 antibody (Herceptin, trastuzumab), but this is an expensive option that not all patients can access and must still be frequently combined with chemotherapy drugs.[2,3]

Protein nanoparticles (PNPs) are increasingly used in a wide variety of biotechnology settings due to their low toxicity, high potential specificity deriving from their polyvalent nature, and low cost. This project focuses on the modification of PNPs to target HER2-positive breast cancer cells for drug delivery. The relative success of this project was determined by flow cytometry and fluorescence microscopy, which confirmed the binding of targeted PNPs and a lack of nonspecific binding. While further experimentation in cytotoxicity and *in vivo* studies is needed, this project presents a novel and successful method of targeting HER2-positive breast cancer cells with PNPs.

Introduction

Cancer treatment is an evolving field with many shortcomings. Amongst them is the balance between cancer destruction and patient health. Systemic cytotoxic drugs have been a common treatment since their discovery, but they are highly toxic to the patient and carry lifelong consequences. Prodrugs have been proposed as a solution to systemic therapy, but targeting is necessary for effective treatment. Protein nanoparticles (PNPs) can not only bind to tumors, but also stabilize the prodrug converting enzymes to preserve their activity. These particles are comprised of proteins that self-assemble into stable nanostructures, which can be chemically or genetically modified to target cells, deliver drugs, or induce apoptosis. Use of PNPs in these settings is increasing in the literature and showing effectiveness.[4] These features qualify PNPs as potential immunotherapeutics against cancer, a disease characterized by unique cell surface receptors.

Taking advantage of the versatility of protein engineering, PNPs can be designed to display antibody-binding domains, such as the Z-domain from *S. aureus*, enabling these PNPs to traffic to cancer cells by receptor-mediated targeting.[5] This project focuses on the modification of protein nanoparticles for drug delivery via targeting against human epidermal growth factor receptor 2 (HER2) positive breast cancer cells. Cancer cells are targeted by exterior modification of protein nanoparticles bearing Z domains, which bind anti-HER2 antibodies. These PNPs are engineered to package cytosine deaminase, a prodrug converting enzyme, which induces cell death by converting 5-flourocytosine, a systemically-administered prodrug, to 5-fluorouricil.

Although diverse in pathophysiology and presentation, cancer is characterized by aberrant growth, avoidance of regulation, and overexpression of growth factors. Breast cancer frequently demonstrates mutated HER2; when mutated, HER2 allows uncontrolled cell division

that results in tumor formation. HER2-positive tumors occur in 15-20% of breast cancer and tend to be more aggressive and less sensitive to treatment than HER2-negative tumors.[2] Herceptin, also known as trastuzumab, is an FDA approved therapy for HER2-positive metastatic breast cancer. This therapy consists of a monoclonal antibody that targets the HER2 receptor to block growth signals from reaching cells overexpressing HER2 and to promote immune response against bound cells.[6] Frequently, Herceptin is coupled with chemotherapy to promote further cell cytotoxicity.[2] However, it is well known that systemic drugs result in dangerous side effects to patients which is why this study proposes combining Herceptin with PNPs packaged with cysteine deaminase and 5-fluorocytosine, a chemotherapeutic prodrug. Herceptin will target PNPs to cancerous cells that are overexpressing HER2 while cysteine deaminase will convert systemically administered 5-fluorocytosine to toxic 5-fluorouracil to promote cytotoxicity of bound cells.

Here, we test the ability of protein nanoparticles encapsidating prodrug-converting enzymes targeted by antibodies to HER2 positive breast cancer to improve targeted killing of tumors. PNPs were assembled from *E. coli* co-transformed with plasmids QB@CD and K46Q-ZZ to synthesize coat protein and prodrug converting enzymes. PNPs were then modified with an anti-HER2 antibody and tagged with green fluorescent protein (GFP). The PNPs could then be analyzed for HER2 targeting ability via flow cytometry and microscopy on cell lines MDA-MB-231, MDA-MB-435, HT-29, and HEK-293. MDA-MB-231, MDA-MB-435, and HT-29 cells express HER2 while HEK-293 is a human embryonic kidney cell line incapable of HER2 expression.[8,9]

Both flow cytometry and microscopy were conducted on all four cell lines to show the ability of anti-HER2 antibody modified PNPs to target HER2-positive cells. Results for both

methods indicate that modified PNPs are effective at specifically targeting the HER2 receptor *in vitro*. Therefore, this method of modifying PNPs is promising as an effective, targeted drug therapy to treat HER2-positive breast cancer.

Materials and Methods

Cloning and Protein Expression

BL21 (DE3) competent *E. coli* cells were co-transformed with two plasmid DNAs (pCDF and pET) to allow for the production of the recombinant coat protein (CP) of the protein nanoparticle, Qß, and prodrug enzyme packaging. This was done by adding a DNA plasmid that contained the Qß CP and selective antibiotic resistance and a DNA plasmid that contained the prodrug enzymes and selective antibiotic resistance then heat shocking the cells. The cells were given an hour to recover then plated on a selective agar plate. After colonies have established, a colony was selected and allowed to inoculate in selective culture media. This culture grew in selective expression media until the correct optical density is measured. The expression culture was treated with ispropyl ß-D-1-thiogalactopyranoside (IPTG) and incubated overnight. The purpose of IPTG is to activate the lac operon in the cells and promote the expression of the Qß protein.[10]

Particle Purification

The Qß particles must then be purified from the cells. The expression cultures were centrifuged to isolate the cells. The pellets were resuspended in Tris buffered saline (TBS, pH: 7.6) and sonicated to lyse the cells and release the Qß protein. The lysate was then centrifuged and the protein was precipitated with ammonium sulfate. This was repeated followed by an extraction with butanol: chloroform (1:1). A final centrifugation resulted in two liquid layers. The upper layer was transferred to a Falcon for further purification. Further purification was conducted using a 10:40 sucrose gradient. The gradients were then imaged using a light column on a fractionator stand after centrifugation for 4 hours. After the particles were extracted, they were then balanced with potassium phosphate buffer (pH: 7.4) and centrifuged for 2 more hours.

The pellet that resulted was then resuspended in potassium phosphate buffer and stored until characterization.

Particle Characterization

After purification, Qß particles needed to be characterized to ensure that the proteins are pure with the expected radii. Methods of characterization included dynamic light scattering (DLS), fast protein liquid chromatography (FPLC), and gel electrophoresis. DLS was performed with an ideal radius of 15 nm known from previous experimentation in the Finn lab. Ideally, the FPLC results would be consistent with the radius observed in DLS and with literature values for elution of Qß PNPs. FPLC is also a good measure of particle purity. The gel was run using a serial dilution with a protein standard. Once the run was complete, the gel was silver stained and showed the expected banding pattern for Qß indicating that the particles are ready for modification.

Particle Modification

Qβ(ZZ)@CD PNPs were labeled with anti-HER2 antibody. Briefly, PNPs were diluted to 0.1 mg/mL and 20 μg PNP was incubated with 50 μg purified anti-human ErbB2/HER2 rat IgG2a (Clone ICR55, Thermo Fisher) for 2 h at 37°C. An antibody: ZZ-domain mole ratio of 0.75 was used for all labeling reactions and was sufficient for uniform labeling of the particles. To serve as a control, the same procedure was repeated with purified mouse IgG1, κ isotype control antibody (Clone MG1-45, BioLegend).

Cell Culture

Four cell lines were cultured: HT-29, MDA-MB-435, MDA-MB-231 as HER2 positive lines and HEK-293 as a HER2 negative line. All lines were brought up from cryopreservation and were cultured in flasks using supplemented media. Flasks were split using Accutase every 2-

4 days depending on the confluence of the adherent cells. When the PNPs were ready for application, cells were removed with Accutase from culture for either flow cytometry or microscopy.

Flow Cytometry

MDA-MB-435, MDA-MB-231, HT-29, and HEK-293 cells were grown to 90% confluence, detached with Accutase, pelleted, and re-suspended in FACS buffer. Approximately 5 x 10^5 cells were aliquoted for each sample. PNPs were added directly to the cell suspensions at a final concentration of 10 nM and incubated at 4°C for 2 h. Cells were washed twice with FACS buffer and fixed with 2% paraformaldehyde for 15 min at room temperature. Following two additional washes, cells were re-suspended in FACS buffer and stored at 4°C until analysis. Cell populations, gated for live cells and 10,000 events, were collected using a BD LSRFortessa cell analyzer (BD Biosciences).

Microscopy

For fluorescence microscopy, cultured cells were imaged on an Eclipse Ti-U fluorescence microscope (Nikon). Prior to imaging, 5x10^5 cells (MDA-MB-435, MDA-MB-231, and HEK-293) were seeded onto 8-chambered microscope slides and allowed to adhere overnight. The following day, cells were incubated for 2 hours at 4°C with 200 μL cell culture media containing anti-HER2 labeled antibody PNPs or unlabeled PNPs. Following incubation, cells were rinsed twice with 1x PBS and fixed with 2% paraformaldehyde for 15 min at room temperature. Cells were subsequently rinsed twice with 1x PBS and imaged immediately. Pictures were taken using NIS Elements imaging software (Nikon) and files were exported and analyzed using ImageJ software.

Results and Discussion

Flow Cytometry

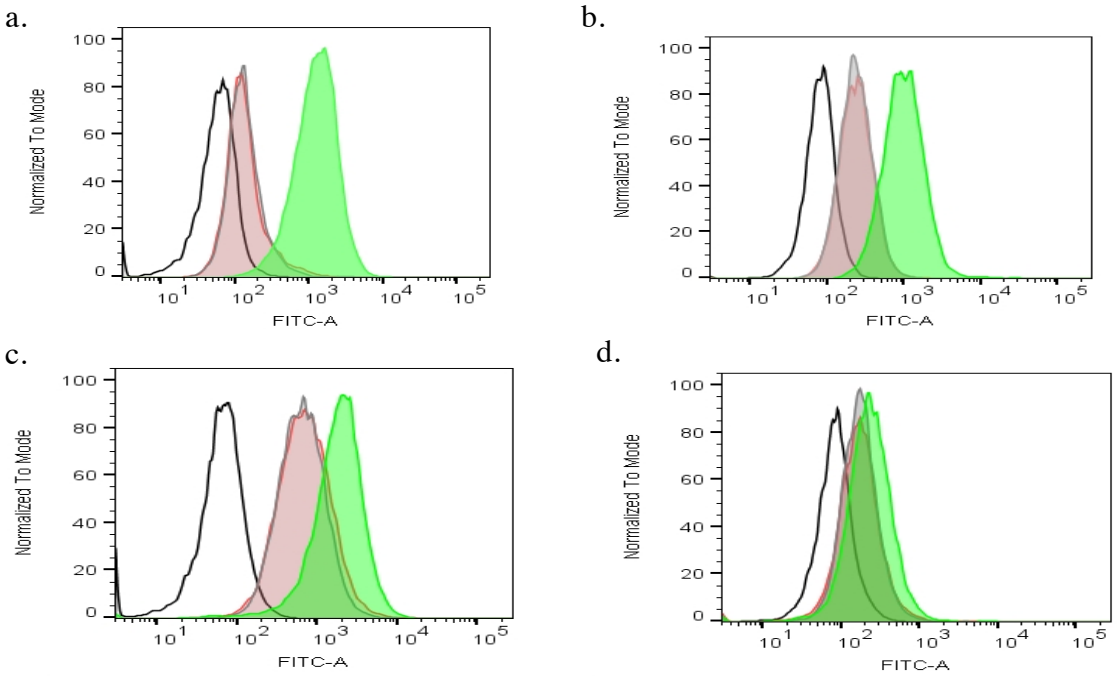

Figure 1. MDA-MB-435 (a), HT-29 (b), MDA-MB-231 (c), and HEK-293 (d) cell line flow cytometry data. Each graph displays the four treatment groups for a single cell line based on fluorescence per cell area (FITC-A). The y-axis is normalized to mode as opposed to count due to the varying numbers of cells in treatment groups. A rightward shift on these graphs indicate the presence of more fluorescent cells. Since the particles are what cause the fluorescence, more fluorescent cells indicate more bound PNPs. The black outlined peaks represented the no particle treatment group, the red peaks represent the unlabeled PNP treatment group, the grey peaks represent the dummy antibody labeled PNP treatment group, and the green peaks represent the anti-HER2 labeled PNP treatment group.

Flow cytometry data on all four cell lines provided promising preliminary data indicating PNPs may be capable of targeting cells which are overexpressing HER2. MDA-MB-435, MDA-MB-231, HT-29, and HEK-293 cells were analyzed via flow cytometry with four experimental groups per cell line. Cells were treated with no particles, unlabeled PNPs, dummy labeled antibody PNPs, or anti-HER2 labeled PNPs. The no particle control group established the baseline reading of the cell line. The unlabeled PNPs control groups determined the "stickiness" of the particles or how much the particles adhere to cells without any type of targeting. The dummy labeled antibody PNPs control group demonstrated whether the antibody targeting used

is specific to the receptor. The anti-HER2 labeled PNPs group served as the experimental group for HER2 receptor targeting.

MDA-MB-435, MDA-MB-231, HT-29 are all HER2-positive cell lines although MDA-MB-231 and HT-29 are only weakly positive. MDA-MB-435 cells showed a marked rightward shift in cell count in the experimental anti-HER2 labeled particle group from the control groups, which is promising for cell targeting potential (Figure 1a). The unlabeled particle and dummy antibody labeled particle groups showed only a slight shift from the background signal indicating a small amount of non-specific binding of particles. HT-29 cells also showed a rightward shift in cell count in the experimental anti-HER2 labeled particle group from the control groups, but the shift is smaller than the shift seen in the MDA-MB-435 cells (Figure 1b). Additionally, this cell type shows a higher amount of non-specific particle binding of unlabeled particles and dummy antibody labeled particles. HT-29 is only weakly HER2 positive so the smaller shift in experimental group is unsurprising. However, the amount of non-specific of binding should be monitored in future cytotoxicity assays. Non-specific binding could pose future problems when the prodrug converting enzymes convert prodrug to toxic bioactive drug. If there is a large amount of non-specific binding, healthy cells will be killed in addition to cancerous cells, which defeats the purpose of this engineered delivery system. MDA-MB-231 cells showed a large rightward shift in cell count in the experimental anti-HER2 labeled particle group from the background control group (Figure 1c). However, there is a large shift of the dummy antibody labeled particle group and unlabeled particle group indicating a large amount of non-specific binding, which again will need to be monitored in cytotoxicity assays.

HEK-293, a HER2 negative cell line used as a control, showed no rightward shift of the experimental group from the control groups (Figure 1d). This result indicates that particles are

not targeting any receptor other than HER2. Additionally, very little non-specific binding is observed in this cell type as indicated by the small rightward shift in unlabeled particle group and dummy antibody group. The results seen in HEK-293 provide further evidence that the shift seen in other cell lines in the experimental treatment group is due to HER2 targeting as opposed to other phenomena. Microscopy results should give further indication whether receptor targeting is occurring or not.

Microscopy

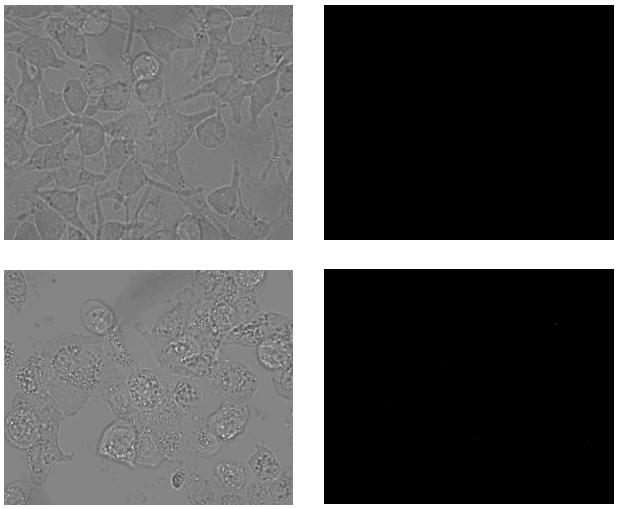

Figure 2. Phase (left) and fluorescent (right) microscopy images of MDA-MB-435 (top) and HT-29 (bottom) cells treated with unlabeled particles. The lack of fluorescence indicates little to no non-specific binding.

MDA-MB-435 and HT-29 were analyzed by fluorescence microscopy. MDA-MB-231 and HEK-293 have yet to be analyzed due to a lack of viable cells in culture, but will be complete soon. Cells were treated with either unlabeled particles or anti-HER2 antibody labeled particles and imaged in both phase and fluorescence. Particles were made fluorescent by tagging them with GFP during antibody modification. Both MDA-MB-435 and HT-29 showed no binding of particles when treated with unlabeled particles as indicated by the lack of fluorescence in microscopy images (Figure 2). This indicates low or nonexistent

levels of non-specific binding, which agrees with the results seen for these cell lines in flow cytometry work (Figure 1). When treated with anti-HER2 labeled particles, both MDA-MB-435 and HT-29 show fluorescent binding of particles around the membranes of the cells (Figure 3). Since both of these cell lines are HER2 positive, this provides further evidence that HER2 is being targeted on the cell membrane by anti-HER2 labeled particles.

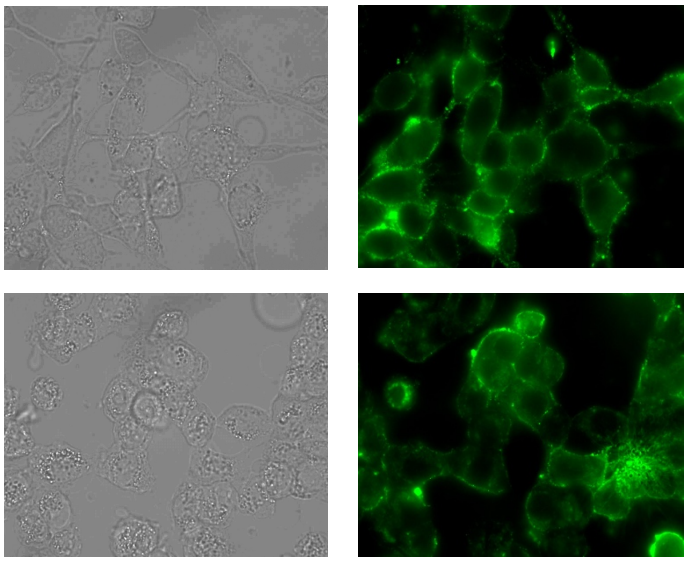

Figure 3. Phase (left) and fluorescent (right) microscopy images of MDA-MB-435 (top) and HT-29 (bottom) cells treated with anti-HER2 labeled particles.

Conclusions and Future Experimentation

Flow cytometry and microscopy indicate that PNPs modified with anti-HER2 antibodies are capable of targeting cells that overexpress HER2. Several experiments are underway to confirm and establish the limits of this technology. First, fluorescence microscopy must be completed with all four cell types to confirm that the particles target all of the HER2-positive cell lines while leaving the HER2-negative cell line unaffected. Measurements of cellular viability (by MTT assay) will then be made, to establish if there is dose-dependent toxicity *in vitro* and to see this correlates with nonspecific or HER2-specific binding. If nonspecific PNP binding is found to induce cell death, the protein nanoparticle can be redesigned to minimize nonspecific association and explore the mechanism of such an effect. The negative cell line used here, HEK-293, is a healthy human embryonic kidney cell line. It will also be useful to test a cancerous HER2-negative cell line to see if any different effects are observed. After establishing the parameters of cellular targeting, prodrug conversion will be engaged to establish the dose dependence of cytotoxicity by this mechanism *in vitro*. These studies will set the stage for testing in an *in vivo* mouse breast cancer model.

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